Functional Analysis of an Eye Enhancer of the *Drosophila eyes absent* Gene: Differential Regulation by Eye Specification Genes

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Genes involved in eye development are highly conserved between vertebrates and *Drosophila*. Given the complex genetic network controlling early eye development, identification of regulatory sequences controlling gene expression will provide valuable insights toward understanding central events of early eye specification. We have focused on defining regulatory elements critical for *Drosophila eyes absent* (*eya*) expression. Although *eya* has a complex expression pattern during development, analysis of eye-specific mutations in the gene revealed a region selectively deleted in the eye-specific alleles. Here we have performed detailed analysis of the region deleted in the eye-specific *eya*^{2} allele. This analysis shows that this region can direct early *eya* gene expression in a pattern consistent with that of normal *eya* in eye progenitor cells. Functional studies indicate that this element will restore appropriate *eya* transcript expression to rescue the eye-specific allele. We have examined regulation of this element during eye specification, both in normal eye development and in ectopic eye formation. These studies demonstrate that the element was activated upon ectopic expression of the eye specification genes *eyeless* and *dachshund*, but does not respond to ectopic expression of *eya* or *sine oculis*. The differential regulation of this element by genes involved during early retinal formation reveals new aspects of the genetic hierarchy of eye development.

Key Words: **eye specification; eye development; *Drosophila*; *eyes absent*; enhancer; *sine oculis*; *dachshund*.**

INTRODUCTION

The *Drosophila* eye presents a powerful system in which to study early events of cell fate specification and differentiation. The fly eye is a highly regular structure composed of approximately 750 individual ommatidial units arranged in a characteristic pattern, making it highly amenable to molecular and genetic analysis (reviewed in Wolff and Ready, 1993; Zipursky and Rubin, 1994; Treisman and Heberlein, 1998). Although early studies focused largely on late events of pattern formation and cell specification, genes functioning early in eye formation have more recently been defined. Study of these genes indicates that, whereas the fly eye is structurally distinct from the vertebrate eye, critical genes that function early in eye formation in *Drosophila* are expressed or known to function in eye development in vertebrates. These genes include *Pax-6* homologues *eyeless* (*ey*) and *twin of eyeless* (*toy*) (Quiring *et al.*, 1994; Czerny *et al.*, 1999), *eya* and the vertebrate *eya* homologues *Eya1*, *Eya2*, and *Eya3* (Bonini *et al.*, 1993; Duncan *et al.*, 1997; Xu *et al.*, 1997; Zimmerman *et al.*, 1997), *dachshund* (*dac*)/*Dach1* (Mardon *et al.*, 1994; Hammond *et al.*, 1998), and *sine oculis* (*so*)/*Six3* (Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994; Oliver *et al.*, 1995).

Since these genes of eye formation are conserved between flies and vertebrates, it has been of interest to define the genetic interactions that lead to eye formation and eventually address the degree of functional conservation of the eye formation pathway. The expression patterns of the genes during the normal eye development and in mutant backgrounds indicate that *toy* expression activates *ey* (Czerny *et al.*, 1999). *ey* expression precedes that of *eya* and *so*, which appear in part dependent upon proper expression of each other, and precedes that of *dac* (Bonini *et al.*, 1997; Chen *et al.*, 1997; Pignoni *et al.*, 1997; Halder *et al.*, 1998). The *Drosophila ey* gene was the first gene shown to be capable of directing ectopic eye formation in the fly upon targeted expression (Halder *et al.*, 1995), indicating that it has the capability to initiate the entire regulatory cascade of genes involved in the complex biological process of eye forma-

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tion. Subsequently, however, both *dac* and *eya* were shown to be capable of directing ectopic eye formation, with *eya* showing synergy in eye formation when combined with *ey, dac,* or *so,* generating larger and more frequent ectopic eyes (Bonini *et al.,* 1997; Chen *et al.,* 1997; Pignoni *et al.,* 1997). *dpp,* a homologue of transforming growth factor-*β,* is also involved in appropriate regulation of eye regulatory pathways and may also function in ectopic eye formation (Chen *et al.,* 1999). The functional redundancy of these genes indicates that, whereas some aspects of the pathway of eye formation might be linear, other aspects have been proposed to involve regulatory loops between the genes (previous references and Desplan, 1997).

Analysis of the elements that control the expression patterns of the genes in eye formation should provide additional details on the genetic hierarchy involved in eye specification, as well as define new genes that are involved in the regulatory cascade. The best defined elements involved in eye development to date are those for the *ey/Pax-6* genes, which show conserved features of regulation in eye formation between vertebrates and flies (Czerny *et al.,* 1999; Hauck *et al.,* 1999; Xu *et al.,* 1999). Analysis of an *ey* gene regulatory element in *Drosophila* has shown that the second *Pax-6* homologue, *toy,* functions prior to *ey* in eye formation to directly activate *ey* expression through this element early in eye formation (Czerny *et al.,* 1999). *ey* may then activate target genes, including *so* and *eya*; recent evidence indicates that *so* is a direct target of *ey* transcriptional regulation (Niimi *et al.,* 1999).

The *eya* gene of *Drosophila* is conserved in vertebrates, defining at least three homologues *Eya1, Eya2,* and *Eya3,* all of which are expressed during development or in the adult eye (Bonini *et al.,* 1993; Duncan *et al.,* 1997; Xu *et al.,* 1997; Zimmerman *et al.,* 1997). Eya function is apparently also conserved, as a mouse *eya* homologue can substitute for the fly gene in *Drosophila* eye formation (Bonini *et al.,* 1997). Although in *Drosophila, eya* is expressed in many tissues in addition to the developing eye, exceptional alleles show highly specific loss of *eya* expression and function in eye progenitor cells (Bonini *et al.,* 1993). Detailed genetic and molecular analysis of these alleles indicated that they are defective in eye-specific regulation of the gene (Leiserson *et al.,* 1994; Zimmerman *et al.,* 1999). Further analysis and study of the mutated region in these alleles, therefore, may reveal new details of the regulation of the *eya* gene during eye formation.

We define here detailed properties of the DNA region affected by the eye-specific *eya* mutations, which appeared to define an eye enhancer element (Zimmerman *et al.,* 1999). We show that this element is necessary, as shown by the eye-specific mutations, and sufficient, as demonstrated by functional studies, to direct expression of *eya* during early stages of eye formation. We address the regulation of this element during normal eye formation and during ectopic eye formation in order to define additional details of the genetic hierarchy involved in eye formation.

MATERIALS AND METHODS

Drosophila stocks. *eya* alleles have been described previously (Bonini *et al.,* 1993, 1998; Leiserson *et al.,* 1994, 1998). Flies were grown on standard cornmeal, molasses media, supplemented with dry yeast. Crosses were performed at 25°C and results were often confirmed at 30°C. Transgenic flies were generated by standard techniques (Rubin and Spradling, 1982) and mapped to the chromosomes following standard protocols. Transgenic lines used were *UAS-eyaI* and *UAS-eyaII* (Bonini *et al.,* 1997, 1998; Pignoni *et al.,* 1997); *UAS-ey* (Halder *et al.,* 1995), *UAS-dac* (Shen and Mardon, 1997), *UAS-so* (Pignoni *et al.,* 1997), and *UAS-hid* (Grether *et al.,* 1995). Additional lines used to determine whether they can activate *eya3-lacZ* expression were *UAS-pnt1, UAS-pnt2, UAS-yan, UAS-hh, UAS-escargot, UAS-dpp, UAS-ras,* and *UAS-Draf* (all obtained from the Bloomington *Drosophila* Stock center).

Generation of enhancer constructs. The *eya-lacZ* transgenes were constructed by using primers 5′ GGATCCAGAGGAGACACTGGC 3′ and 5′ TGATCAATTAACTGACCTGCTCAACTC 3′, to amplify the 322-bp sequence corresponding to the eya^2 deletion region, incorporating *Bam*HI and *Bcl*I restriction sites at the 5′ and 3′ ends, respectively. The 322-bp fragment was cloned into the pCRII vector (Invitrogen) and sequenced to insure fidelity. The 322-bp sequence was excised as a *Bam*HI–*Bcl*I fragment and inserted into the *Bam*HI and *Bcl*I sites within the polylinker of pSL1190 (Stratagene). Concatamers of either three or six tandems repeats were constructed by repeated subcloning of the 322-bp *Bam*HI–*Bcl*I fragment into the *Bcl*I site of the pSL1190 vector containing the previous 322-bp subclone. Fragments containing three or six tandem repeats were excised from pSL1190 and cloned upstream of the *hsp43* minimal promoter and the full-length *β-galactosidase* gene of the pCasper-hs43-*β*gal vector (Thummel *et al.,* 1988). This yielded *eya3-lacZ* and *eya6-lacZ* transgenes, with the *eya* element in the forward (5′ to 3′) orientation relative to the normal orientation of the *eya* gene, and the *eya3B-lacZ* transgene, with the element in the reverse orientation. For construction of the *eya-GAL4* transgene, a fragment containing three tandem copies of the *eya* element in pSL1190 was excised as a *Bam*HI–*Eco*RV or *Kpn*I–*Bam*HI fragment and cloned upstream of the GAL4 open reading frame of pGaTB (Brand and Perrimon, 1993) into the *Bam*HI–*Pvu*I or *Kpn*I–*Bam*HI sites, respectively. This gave rise to the *eya-GAL4* transgene with the enhancer element in either forward or reverse orientation, respectively, within pGaTB. The *eya-GAL4* transgene was created by excising the *eya-GAL4* fragment from pGaTB as a *Kpn*I–*Not*I fragment and inserting into the *Kpn*I–*Not*I sites of the pCasper4 transformation vector (Thummel *et al.,* 1988). The sequence of the *eya* 5′UTR, which includes the enhancer region, is GenBank Accession No. AF190902.

Immunocytochemistry and histology. Immunostaining was performed was described (Bonini *et al.,* 1997). Primary antibodies were anti-EyaMab10H6 (1:10, Bonini *et al.,* 1998), anti-Elav (1:5, O'Neil *et al.,* 1994), anti-*β*-Gal (1:1, Developmental Studies Hybridoma Bank; and 1:50, ICN Biomedicals, Aurora, OH). All secondary antibodies were from Jackson ImmunoResearch Laboratories, conjugated to fluorescein or Texas red. Confocal microscopy was performed on a Leica Model TCS SP ultraviolet and visible confocal imaging spectrophotometer microscope. Staining to detect *β*-galactosidase expression was performed as described (Hiromi and Gehring, 1987). Studies to detect *β-galactosidase* gene expression by *in situ* were performed as described (Bonini *et al.,* 1993).

TABLE 1
***eya* Enhancer Element Constructs**

Construct	Eye disc staining	Additional comments
eya3.lacZ		
→→→lacZ	+	
eya3B.lacZ		
←←←lacZ	++	
eya6.lacZ		
→→→→→→lacZ	+++	
eya-GAL4		
→→→GAL4		Rescues *eya*^{2} with *UAS-eya*;
	+++	phenocopies *eya* mutant with *UAS-hid*

Note. Arrow represents the *eya*^{2} deletion; pointing to the right it indicates a 5′ to 3′ orientation and pointing to the left it indicates a 3′ to 5′ orientation relative to the normal *eya* gene. The first three constructs were ligated to a minimal promoter upstream of the coding region for *β-galactosidase.* The last construct was made in the GAL4 vector pGaTB and expression visualized by crossing transgenic flies to a fly line bearing *UAS-lacZ.*

RESULTS

The Region Defined by Eye-Specific eya Mutations Directs Expression to Eye Progenitor Cells

During eye formation, the Eya protein is initially expressed anterior to the morphogenetic furrow, where it plays a critical role in eye formation (Bonini *et al.*, 1993). Eya expression is maintained posterior to the furrow as development progresses across the eye disc. Eya is also expressed in the cells that give rise to the ocelli. In the eye-specific *eya*^{1} and *eya*^{2} mutants, Eya expression is selectively lost from the compound eye field, but is maintained in ocellar progenitor cells (Bonini *et al.*, 1993; Leiserson *et al.*, 1998). Molecular analysis of these mutants revealed that they are overlapping deletions upstream of the start of transcription, with the *eya*^{2} mutation defining a small 322-bp region (Zimmerman *et al.*, 1999). These results suggested that this region defines a regulatory element for eye-specific transcription of the *eya* gene, as it is affected in two independently isolated alleles of the *eya* gene whose phenotype is restricted to loss of the adult compound eye. Analysis of transformation constructs with three or six tandem repeats of the fragment deleted in the *eya*^{2} allele, upstream of a minimal promoter and the coding region of the reporter gene *β-galactosidase,* confirmed that the region defined by the *eya*^{2} deletion can direct gene expression in the pattern of the *eya* gene in eye progenitor cells (Table 1, Fig. 1). We found no significant staining elsewhere in the animal (Zimmerman *et al.*, 1999), indicating that expression was specific for the eye portion of the eye-antennal imaginal disc.

Here, we performed a detailed analysis of the temporal expression pattern in the developing eye disc. These studies showed that we could detect expression as early as the first instar larval period, continuing through the second to the third instar larval periods (Figs. 1A–1C). The expression pattern in first and second larval instar eye discs appeared uniform, covering the entire eye disc. Expression was not detected in the eye anlagan of the embryo (data not shown). These data indicated that the region defined by the *eya*^{2} deletion region functioned as an eye enhancer that directs expression selectively to the developing eye field from early stages of eye formation.

Additional studies suggested that the expression of the enhancer appeared limited to prior to the furrow. In mid third instar larvae, expression was restricted to the eye-antennal disc. Staining was strong prior to the furrow in young third instar larval discs, but became increasingly weak as discs matured, such that in older discs, we typically failed to detect enhancer expression by antibody staining. Because the expression after the furrow may represent perdurance of the β-galactosidase protein, we performed *in situ* analysis with the *β-galactosidase* gene. *β-galactosidase* expression was detectable in young discs, but not in older discs (Figs. 1H and 1I), consistent with enhancer expression being limited to prior to the morphogenetic furrow. The expression pattern by *in situ* appeared the same as that of *β-galactosidase* driven by the *eyeless* regulatory element (data not shown), which selectively expresses prior to the furrow (Quiring *et al.*, 1994).

The eya Eye Enhancer Restores Eye Development to eya Mutants

If the *eya* element were sufficient for *eya* expression in eye progenitor cells, then the *eya* cDNA driven in this expression pattern should rescue the eye-specific *eya* mutations. To test this, a construct was made inserting three tandem copies of the *eya*^{2} deletion region into the GAL4 vector pGaTB (Brand and Perrimon, 1993), creating an *eya enhancer*-GAL4 construct designated *eya*-GAL4. Using the GAL4/UAS expression system (Brand and Perrimon, 1993), we addressed functional aspects of the enhancer region by crossing these transgenic fly lines to lines bearing various *UAS* transgenes. We confirmed that *eya-GAL4* directed expression of the reporter transgene *UAS-lacZ* in an expression pattern similar to those of the constructs previously examined (Table 1). Double labeling with the neural marker Elav confirmed that expression occurred before neuronal differentiation (Figs. 1D–1F). Examination of eye imaginal discs from earlier developmental stages indicated that expression was similar to that described above (data not shown).

To address functional aspects of the *eya* enhancer, we crossed flies bearing *eya-GAL4* to those bearing the *UAS-eya* transgene in the *eya*^{2} mutant background. These data indicated that the *eya-GAL4* transgene was indeed functional in the *eya*^{2} mutant and directed expression of the *eya* cDNA to partially rescue the mutant phenotype (Fig. 2).

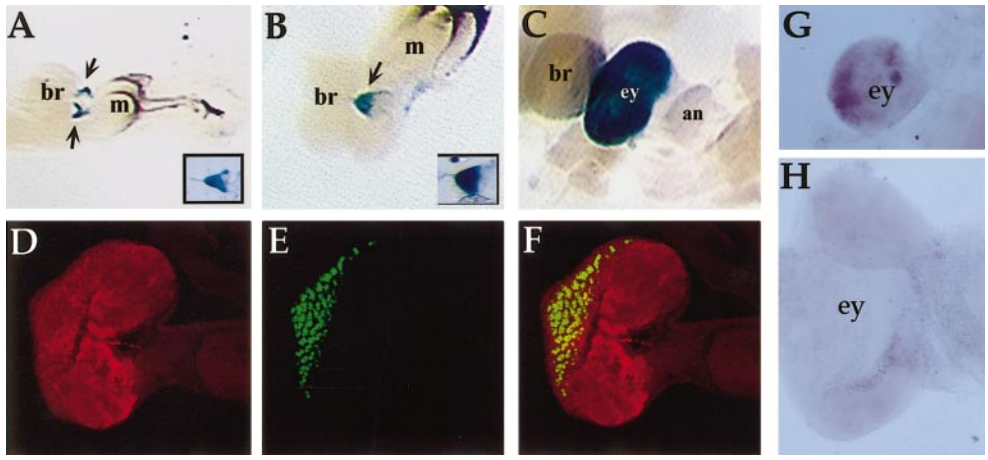


FIG. 1. Expression pattern of the *eya* eye enhancer element. Expression of the *eya* enhancer is activated in the eye disc. (A–C) Staining for *β-galactosidase* activity with a histochemical stain. Expression is detected as early as the first instar larval period (A) and remains on during the second (B) and third larval instar periods (C). Larvae bearing the *eya6-lacZ* transgene. (D–F) Confocal images of the expression pattern of the *eya-GAL4* transgene in the developing eye disc, visualized by crossing to a *UAS-lacZ* transgenic line. β-Galactosidase antibody pattern (red, D) and Elav antibody expression pattern (green, E) and overlay of the two patterns (F). The expression pattern of Elav highlights the developing neurons posterior to the furrow, confirming that the *eya* enhancer is activated prior to the furrow. (G, H) *In situ* expression pattern of the *β-galactosidase* gene in the *eya6-lacZ* transgenic line. Expression can be detected early in third instar larval eye discs (G), but not later (H). These data suggest that enhancer expression is activated selectively ahead of the furrow; residual lacZ protein expression observed after the furrow would appear to represent perdurance of lacZ. m, mouth hooks; br, brain; an, antennal portion; and ey, eye portion of the eye-antennal imaginal disc.

Thus, the transgene restored appropriate DNA regulatory sequence to the eye-specific mutant in order to direct and restore *eya* expression in eye formation.

We also addressed whether we could mimic the *eya* mutant phenotype by directing expression of the cell death gene *hid* in the *eya* expression pattern. Loss of *eya* function leads to increased cell death anterior to the furrow (Bonini *et al.*, 1993), suggesting that the cells are dying by programmed cell death early in eye formation. Northern blot analysis confirmed that the cell death genes *hid* and *reaper* were upregulated in eye discs of the eya^1 eye-specific mutant (Fig. 3A), consistent with the cell death observed in *eya* mutant eye discs occurring through molecularly defined apoptotic pathways. Moreover, normal flies bearing *eya-GAL4 UAS-hid* indeed mimicked *eya* mutants, showing a reduced eye phenotype resembling that of severe alleles of the *eya* gene (Figs. 3C and 3D; Bonini *et al.*, 1993). These flies showed no phenotype other than a reduced eye, confirming specificity of the eye enhancer to eye progenitor cells.

Functional Regulation of the eya Enhancer by Eye Developmental Genes

Having defined a regulatory element that was both necessary and sufficient, at least in part, for eye-specific transcription of *eya*, we addressed regulation of the *eya* enhancer by known genes critical to early events of *Drosophila* eye formation. First, we addressed whether the enhancer was expressed in mutants of *ey*, *so*, and *dac*. *ey* initiates expression prior to *eya*, and normal *eya* expression is dependent upon *ey* gene activity (Bonini *et al.*, 1997; Halder *et al.*, 1998). In so^1 mutants, *eya* expression is present, but appears reduced (Pignoni *et al.*, 1997; Halder *et al.*, 1998). In *dac* mutants, *eya* is expressed (Chen *et al.*, 1997). Thus, we were interested to determine whether expression of the eye-specific regulatory region occurred in these mutants, to address the degree to which the element reflected known regulation of Eya. To perform these stud-

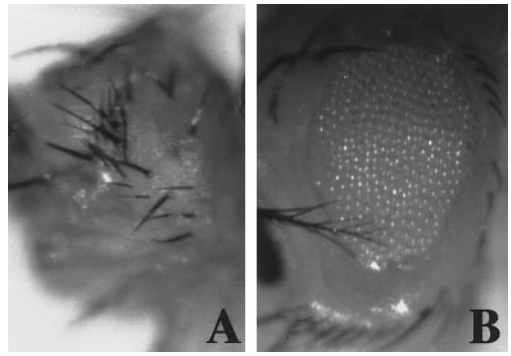
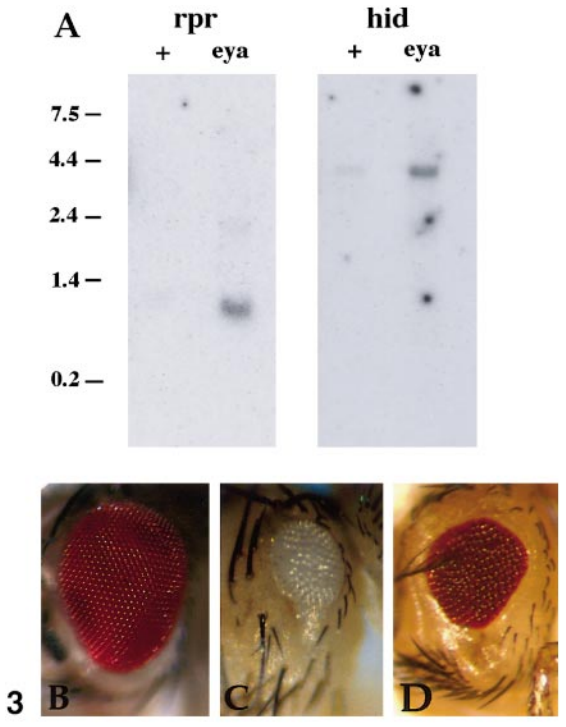


FIG. 2. Functional rescue of the *eya* eye-specific mutant with the *eya* enhancer element. (A) The eya^2 mutant. (B) Restoration of the eye to the eya^2 mutant, using the *eya* enhancer element to drive expression of the *eya* cDNA. Genotype eya^2; *eya-GAL4/UAS-eya.*



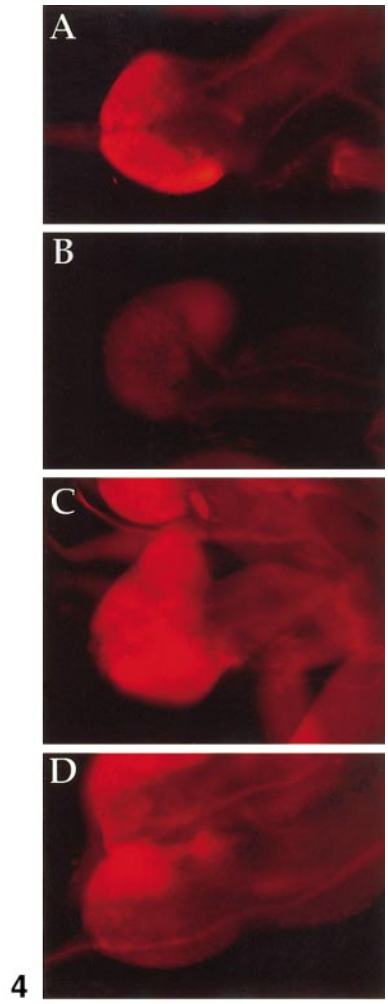


FIG. 3. Expression of the *hid* killer gene by the *eya* enhancer element phenocopies *eya* mutants. (A) Northern blot expression of the cell death genes *hid* and *reaper* in normal eye-antennal disc preparations (left column of each blot) and in those of the mutant eya^{1} (right column of each blot). Whereas normally both *hid* and *rpr* are expressed at low levels, expression was greatly increased in the developing eye tissue of the eya^{1} mutant, which has greatly increased cell death. (B–D) Expression of the cell death gene *hid* by the *eya* eye enhancer phenocopies *eya* mutants. (B) A wild-type eye. (C) Expression of the potent killer gene *hid* by the *eya* enhancer element. The eye is greatly reduced, similar to the phenotype of severe alleles of the *eya* gene (D; also Bonini *et al.*, 1993). No other phenotypes were observed, consistent with the specificity of the *eya* enhancer element to eye progenitor cells. (D) Reduced eye phenotype of a severe *eya* allelic combination, eya^{E1}/eya^{1}.

FIG. 4. *eya* enhancer expression in *ey*, *dac*, and *so* mutant eye discs. β-Galactosidase expression of the *eya3-lacZ* transgene in early third instar larval eye discs from (A) normal larvae and (B) ey^{2} mutant, (C) dac^{3} mutant, and (D) so^{1} mutant larvae. Expression of the *eya* enhancer is missing in *ey* mutant eye discs (B). This suggests that enhancer expression is dependent upon *ey* gene activity. In the *dac* mutant, enhancer expression is strong (C). In *so* mutant eye discs (C), enhancer activation occurred, but was reduced. This may reflect a dependence upon *so* gene activity or an apparent decrease in expression due to loss of cells through cell death in *so* mutant eye discs.

ies, we crossed the *eya3-lacZ* transgene into ey^{2}, so^{1}, and dac^{3} mutant allele backgrounds and then stained eye discs for β-galactosidase activity (Fig. 4). The enhancer remained expression in so^{1} and dac^{3} mutant eye discs, but expression appeared defective in ey^{2} mutants (Fig. 4). This suggested that, like normal *eya* gene expression, the activity of this enhancer was dependent upon *ey* activity, but appeared independent of *dac* and, at least in part, independent of *so* gene function. These data suggested that the enhancer element was regulated in a manner consistent with known regulation of Eya expression in the eye.

Next, we addressed regulation of the *eya* enhancer upon directed expression of the *ey*, *dac*, and *so* genes and the latter in combination with *eya*. Current views of the

regulatory pathway of eye formation propose that these genes are connected in regulatory loops, such that the expression of all becomes directed by the others during ectopic eye formation (Bonini *et al.*, 1997; Chen *et al.*, 1997; Desplan, 1997; Pignoni *et al.*, 1997; Czerny *et al.*, 1999). This model predicts that the *eya* enhancer should be expressed upon ectopic eye formation when directed by any of these genes.

Using the GAL4/UAS expression system, we directed the expression of these genes by the *dpp-GAL4* driver in a genetic background containing the *eya6-lacZ* reporter construct. Normally, *eya* enhancer activity, like *eya* gene expression, is restricted to the eye disc and is not present in the antennal portion of the disc. When driven by *dpp-GAL4*, these transgenes or combinations thereof—with the exception of *UAS-so* alone—will direct ectopic eye development in the antennal portion of the disc. We therefore stained eye-antennal discs of larvae bearing these different transgene combinations for β-galactosidase expression. In order to correlate the expression of the *eya* enhancer with ectopic eye development directed by these genes, we also double labeled eye-antennal discs in order to detect *eya* enhancer activity with β-galactosidase protein expression, and ectopic eye formation with an antibody to the neural-specific protein Elav, as a marker of ectopic photoreceptor development. These studies revealed that ectopic expression of the enhancer was detectable upon ectopic expression of *ey* and *dac*, but not of *eya* or *eya* in combination with *so* (Figs. 5 and 6).

As anticipated by previous studies, we found that *UAS-ey* indeed activated expression of the enhancer in the antennal portion of the eye-antennal imaginal disc (Figs. 5B, 6A, and 6B). In these animals, ectopic eye formation was also observed (Fig. 6C). We also found that *UAS-dac* activated expression of the *eya* enhancer in the region undergoing ectopic eye formation in the antennal disc (Figs. 5C and 6D–6F). However, the *eya* gene itself failed to activate expression of the enhancer, even though Elav staining confirmed ectopic retinal development in the antennal disc, and adult animals displayed ectopic eyes (Figs. 5A and 6G–6I). Upon combination of *UAS-dac* and *UAS-eya*, which synergize in ectopic eye formation (Chen *et al.*, 1997), the enhancer responded as it did to *UAS-dac* expression alone, becoming activated (Fig. 5D). This indicated that *eya* did not negatively affect the *dac*-mediated response. *UAS-so* on its own fails to induce ectopic eye formation in *Drosophila* (Pignoni *et al.*, 1997) and failed to activate expression of the *eya* enhancer (data not shown). Moreover, even upon coexpression of *UAS-eya* and *UAS-so*, which synergize in ectopic eye formation (Pignoni *et al.*, 1997), no ectopic expression of the enhancer was observed (Figs. 5E and 6J–6L). These data indicate that the *eya* enhancer region displays differential regulation by eye specification genes.

The *eya* enhancer contains potential binding sites for Ets transcription factors (Zimmerman *et al.*, 1999). The enhancer also has a potential binding site for Mad, the transcription factor involved in *dpp* pathway signaling (Kim *et al.*, 1997), and *dpp* plays a role during ectopic eye formation (Chen *et al.*, 1999). We therefore tested the ability of other *UAS* transgenes to activate expression of the *eya* enhancer, including *UAS-dpp* and the Ets transgenic lines *UAS-pointed* and *UAS-yan*. However, ectopic enhancer expression was not observed with other tested lines (data not shown; see Materials and Methods).

DISCUSSION

We describe functional regulation of a region of the *eya* gene that can direct expression to eye progenitor cells. This *eya* enhancer was sufficient to drive the expression of an *eya* cDNA transgene and functionally rescue, at least in part, an eye-specific *eya* deletion mutation, indicating that this domain can direct appropriate *eya* expression early in eye formation. Greater analysis of this domain indicated that it responds differentially to eye specification genes, providing new insight into the molecular genetic pathways of eye formation. The enhancer responded to ectopic expression of *ey* and *dac*, but failed to become activated upon eye formation directed by *eya* or *eya* plus *so*. These results suggest that the molecular mechanisms by which *ey* and *dac* direct ectopic eye formation are at least in part distinct from those of *eya* and *so*.

The Eye-Specific Alleles of eya Define an Eye Enhancer Element

The *Drosophila* compound eye develops from a monolayer epithelium (reviewed in Wolff and Ready, 1993; Zipursky and Rubin, 1994; Treisman and Heberlein, 1998). During the third instar larval period, differentiation proceeds as a wave from posterior to anterior across the eye disc epithelium. The leading edge of this wave of differentiation is referred to as the morphogenetic furrow. Cells ahead of the furrow are actively dividing and undifferentiated, whereas cells behind the furrow have stopped dividing and are actively recruited into the neural clusters that eventually give rise to the ommatidial units of the compound eye. During eye development, Eya expression is found in all eye progenitor cells ahead of the furrow, beginning during the second larval instar (Bonini *et al.*, 1993). As the wave of differentiation proceeds, Eya expression is maintained in select cells posterior to the furrow, where Eya plays a role in later events of neural development (Pignoni *et al.*, 1997).

Our results demonstrate that the region deleted in the eye-specific alleles of *eya* defines an eye enhancer. The 322-bp region deleted in the eya^2 mutant is necessary for eye expression, as this deletion mutation leads to complete loss of *eya* expression in the eye progenitor cells (Bonini *et al.*, 1993; Leiserson *et al.*, 1998). This region is also sufficient for eye progenitor cell expression, because it targets gene expression to eye progenitor cells (see Fig. 1). The

domain of expression of the eye enhancer appears generally consistent with that previously described for Eya protein prior to furrow formation. Activation of the *eya* enhancer element was detected as early as the first instar larval period, which is prior to the time when Eya protein is first detectable (Bonini *et al.,* 1993). However, detection of the reporter gene may be more sensitive and stable than that of Eya protein, which is difficult to detect at early stages. The functionality of this enhancer was revealed by demonstrating that driving expression of an *eya* transgene or a cell death gene by this enhancer region could partially rescue an eye-specific *eya* mutant and partially delete the eye in wild-type flies to phenocopy *eya* mutants, respectively. Our data suggest that the enhancer reflects expression of *eya* prior to the furrow. Although LacZ expression could be detected after the furrow, this appears to reflect perdurance of LacZ protein because *in situ* analysis of *β-galactosidase* expression suggests that expression was detectable only in early third instar larval eye tissue, but not in later third instar eye discs, when the furrow has progressed. Based on these data, this region defines a regulatory region critical for early expression of the *eya* gene in eye progenitor cells. We subsequently used this region to address greater molecular genetic control of regulatory pathways of *eya* gene control in eye development.

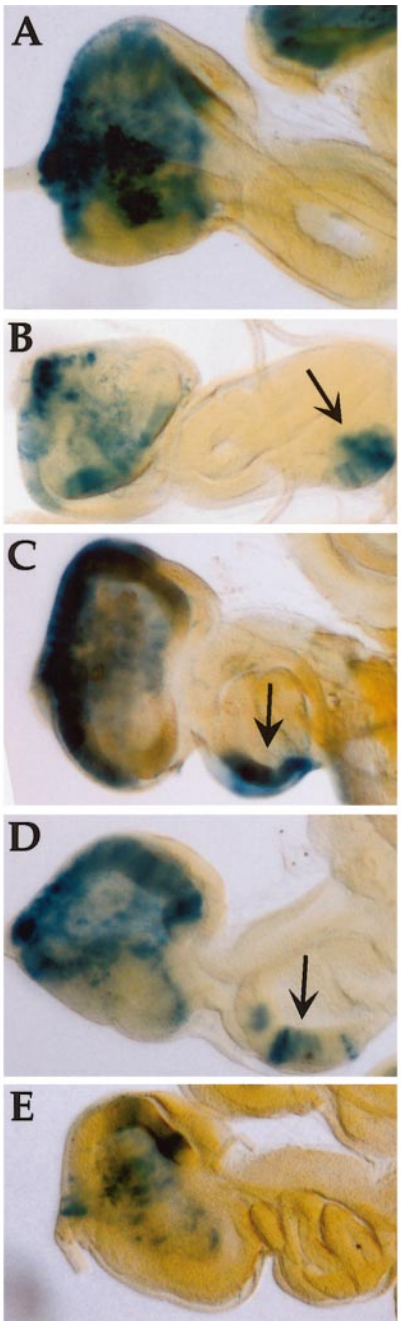
Differential Regulation of the eya Enhancer upon Ectopic Eye Formation

The *eya* enhancer is expressed in *ey, so,* and *dac* mutant eye discs in a pattern consistent with previous studies of Eya protein expression during normal eye development (Bonini *et al.,* 1997; Halder *et al.,* 1998). Normally, *eya* expression is dependent upon *ey* activity, partially dependent upon *so* activity, and independent of *dac* activity (Bonini *et al.,* 1997; Chen *et al.,* 1997; Desplan, 1997; Pignoni *et al.,* 1997; Halder *et al.,* 1998; Czerny *et al.,* 1999). We then addressed regulation during ectopic eye formation in order to define genes that control the expression of this *eya* enhancer region and observed differential activation of the *eya* enhancer. Activity of the enhancer was detected upon *ey*- and *dac*-induced eye formation, as anticipated by previous studies. However, enhancer activation was not apparent upon ectopic *eya* or *so* gene expression or the combination of *eya* and *so* together. Thus, this *eya* enhancer appears to be selectively activated during ectopic eye formation, indicating a molecular distinction in how *ey* and *dac* genes induce ectopic retinal tissue compared to that of the *eya* and *so* genes, at least with respect to regulation revealed by this element.

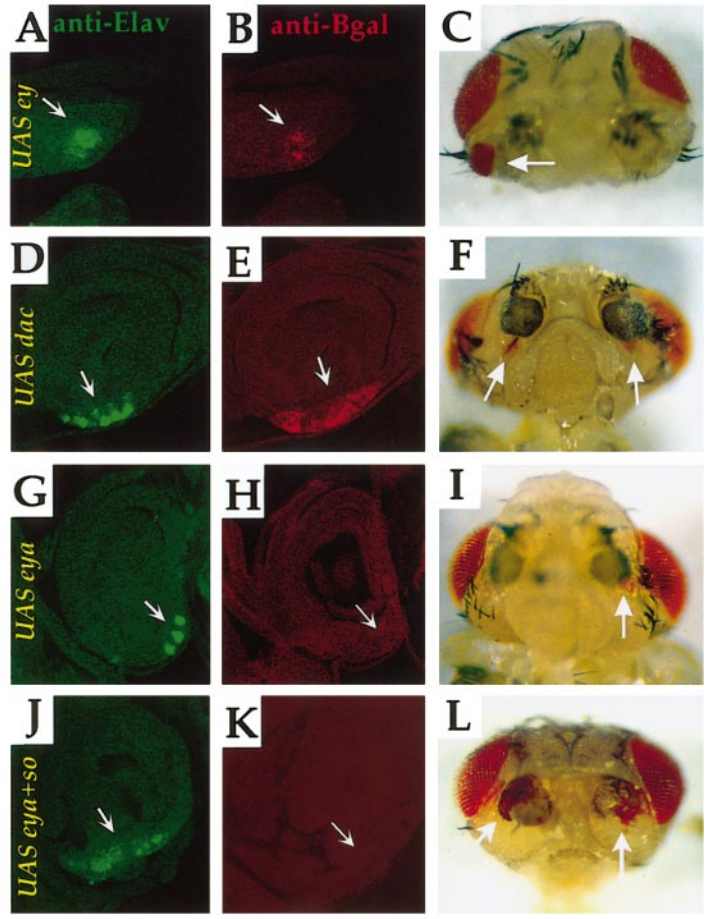
Current models on the regulation of early retinal specification suggest that *ey, eya, so,* and *dac* function in a combinatorial manner to implement the eye developmental program (Bonini *et al.,* 1997; Chen *et al.,* 1997; Desplan, 1997; Pignoni *et al.,* 1997; Czerny *et al.,* 1999). The genetic pathways governing these events have been suggested to involve feedback loops such that all genes regulate the expression of each other. The *Pax-6* homologues *toy* and *ey* occupy the top of this genetic hierarchy, with *ey* being a direct target of *toy* (Czerny *et al.,* 1999). Expression studies place *eya, so,* and *dac* downstream of *ey,* with *so* being a direct target of *ey* transcriptional activity (Bonini *et al.,* 1997; Chen *et al.,* 1997; Pignoni *et al.,* 1997; Halder *et al.,* 1998; Niimi *et al.,* 1999). *eya* and *so* appear to be partially dependent upon each other. *dac* expression has been interpreted as being fully dependent upon *eya* function (although the data indicate that at least some Dac expression remains in *eya* mutant eye discs; see Chen *et al.,* 1997, and below). Given the genetic and biochemical data that suggest that Eya can synergize and physically interact with So and Dac proteins, it has been proposed that, once activated, these gene products function in a concerted manner to autoregulate and direct downstream components of eye development (Desplan, 1997; Chen *et al.,* 1997; Pignoni *et al.,* 1997).

The regulation of this defined eye enhancer for *eya* suggests that *eya* and *so* function at least in part distinctly from *dac* and *ey* in ectopic eye formation. Whereas *ey* and *dac* either directly activate or feedback to activate *eya* expression, *eya* and *so* do not participate in regulatory loops to the level of activation of *eya* gene expression as defined by this enhancer. The *eya* and *so* gene functions in the eye appear strikingly similar not only in the adult, but also in the developing eye disc (Bonini *et al.,* 1993; Cheyette *et al.,* 1994; Serikaku and O'Tousa, 1994; Pignoni *et al.,* 1997). This, together with functional synergy between the two in eye formation, and a demonstrated physical interaction between the two proteins *in vitro* supports the notion that the two indeed function as (or in) a complex in eye formation. Moreover, at the level of activation of this defined *eya* enhancer, *eya* acts the same as *eya* and *so* together—that is, it fails to activate expression of the enhancer. That the enhancer can restore eye development to *eya* mutants null for early gene expression in the eye primordia (Leiserson *et al.,* 1998) also indicates that *eya* is not required for activation of the enhancer. That directed expression of *so* fails to induce ectopic eye formation in *Drosophila* (Pignoni *et al.,* 1997) may be due, in part, to the inability of *so* to activate expression of the *eya* gene—an essential gene for eye specification and a property shared by both *ey* and *dac*, both of which can direct ectopic eye formation.

eya can also synergize with *dac* in ectopic eye development, and physically interacts with the Dac protein (Chen *et al.,* 1997). The loss-of-function phenotype of *dac* in the eye, however, is not identical to that of *eya* and *so* (Mardon *et al.,* 1994). These studies also suggest that *dac* is not acting the same way as *eya* with respect to this molecular indicator of the *eya* enhancer—*dac* strongly activated expression, but *eya* did not. *dac* has previously been placed downstream of *eya* due to expression studies (Chen *et al.,* 1997). However, Dac is reduced, but not missing from *eya* mutant eye discs (see Chen *et al.,* 1997; also G. Gray-Board and N. Bonini, unpublished). The reduced expression may reflect massive loss of eye progenitor cells in *eya* mutant



5



6

FIG. 5. Selective activation of the *eya* enhancer upon directed gene expression. Eye-antennal discs stained for β-galactosidase activity to detect ectopic activation of the *eya* enhancer, with the *eya6-lacZ* transgene. (A) Ectopic expression of *eya* failed to induce detectable activation of the *eya* enhancer in the antennal disc. (B–E) Ectopic expression of (B) *ey* and (C) *dac* resulted in ectopic activation of the enhancer in the antennal disc (arrow). Expression of (D) *dac* and *eya* acted like *dac* only, with ectopic enhancer activation, indicating that *eya* was not negatively regulating the *dac* response. (E) *eya* and *so* failed to activate ectopic expression of the enhancer. Typically, enhancer expression in the eye disc appeared weaker in this gene combination. Flies of genotype *w*; *dpp-GAL4 eya6-lacZ* in *trans* with the appropriate *UAS-transgene* (*UAS-ey*, *UAS-dac*, or *UAS-eya* or recombinant lines of *UAS-dac UAS-eya*, or *UAS-eya UAS-so*).

FIG. 6. Selective expression of the *eya* eye enhancer upon ectopic eye formation. Confocal images (columns 1 and 2) and dissecting microscope images (column 3) of ectopic gene expression and ectopic eye formation upon targeted expression of specific genes. Elav expression highlights ectopic retinal tissue (column 1, detected with an antibody to Elav and visualized with a fluorescein-conjugated secondary antibody). β-Galactosidase expression indicates *eya* enhancer activity (column 2, antibody to β-galactosidase, visualized with a Texas red-conjugated secondary antibody). (A–C) *dpp-GAL4*-driven expression of *UAS-ey* in the antennal disc induced ectopic retinal

eye discs; alternatively or in addition, there may be a partial dependence of Dac expression upon *eya* gene function. Thus, Dac may indeed be involved normally in aspects of *eya* gene expression. Previous studies showing Eya expression upon ectopic eye formation are confounded by the fact that Eya is expressed both prior to and after the furrow, but this expression is likely to be under the control of different regulatory elements. The element defined here presents a probe for at least some aspects of the early regulation of *eya* gene expression. The functional requirement by *eya* for *ey* and *dac* activity (and vice versa) in ectopic eye formation may reflect concurrent roles or other, later roles of these genes in eye formation. *ey* clearly has multiple roles at distinct times in eye development, such as regulation of genes important for late events of photoreceptor cell differentiation (Sheng *et al.*, 1997), in addition to the early function stressed here.

With respect to *eya* enhancer activation, *ey* and *dac* may directly bind to the *eya* eye enhancer or the regulation may be indirect through additional, yet-to-be defined genes. We suggest the regulation may not be direct, at least for Ey, as Ey binding sites are not clearly apparent within the element (Zimmerman *et al.*, 1999). Whether Dac protein directly binds to DNA is yet to be determined, but it likely interacts with known transcriptional regulators in addition to interacting with Eya (Hammond *et al.*, 1998). Yeast one-hybrid experiments have also failed to support direct activation of the *eya* enhancer by Dac or Ey (as well as confirmed lack of activation by Eya and So; Q. Bui and N. Bonini, unpublished).

These studies provide a framework from which to define additional molecular genetic controls on early retinal specification. Recent studies showing that the fundamentals of *ey/Pax-6* regulation can cross species boundaries (Xu *et al.*, 1999) suggests that not only are elements of the genetic pathway controlling eye development conserved in vertebrates, but fundamental aspects of the regulatory mechanisms may also be conserved. Given that vertebrate Eya homologues display functional rescue of *Drosophila eya* mutants (Bonini *et al.*, 1997), key regulatory aspects of *eya* gene expression, in addition to the function of the protein, may also be conserved. Eya is a critical gene of eye formation, with complex regulation of expression as shown here, as well as complex protein interactions (Chen *et al.*, 1997; Pignoni *et al.*, 1997), and multiple downstream targets (Hazelett *et al.*, 1998). This eye enhancer controlling early *eya* expression provides a molecular genetic tool to help dissect additional regulatory events of eye specification that are involved in the conserved pathways of eye formation.

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development detected with an antibody to Elav (A), leading to the eventual formation of ectopic compound eyes in the adult (C, arrow). Staining for β-galactosidase showed activation of the *eya* enhancer (B). Larvae and flies of genotype *w*; *dpp-GAL4 eya6-lacZ/UAS-ey*. (D–F) *dpp-GAL4*-driven expression of *UAS-dac* in the antennal disc induced ectopic retinal development detected by Elav antibody staining (D), leading to the formation of ectopic compound eyes in the adult head (F, arrow). β-Galactosidase antibody staining revealed activation of the *eya* enhancer (E). Larvae and flies of genotype *w*; *dpp-GAL4 eya6-lacZ/UAS-dac.* (G–I) *dpp-GAL4*-driven expression of *UAS-eya* in the antennal disc induced ectopic retinal development detected by Elav antibody (G), leading to the formation of ectopic compound eyes in the adult (I, arrow). However, ectopic β-galactosidase expression was not detected in the antennal disc (H), indicating that the *eya* enhancer was not activated. Flies of genotype *w*; *dpp-GAL4 eya6-lacZ/UAS-eya.* (J–L) *dpp-GAL4*-driven expression of *UAS-eya* and *UAS-so* in the antennal disc induced ectopic retinal development detected by Elav antibody staining (J), leading to the formation of ectopic compound eyes in the adult (L, arrow). However, ectopic β-galactosidase expression was not detected in the antennal disc (K), indicating that the *eya* enhancer was not activated. Flies of genotype *w*; *dpp-GAL4 eya6-lacZ/UAS-eya UAS-so.*

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